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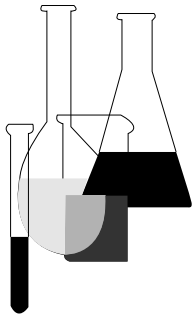
Health Effects Test Guidelines OPPTS 870.6100 Acute and 28-Day Delayed Neurotoxicity of Organophosphorus Substances



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

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OPPTS 870.6100 Acute and 28-day delayed neurotoxicity of organophosphorus substances.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background.** The source material used in developing this harmonized OPPTS test guideline are 40 CFR 798.6450 NTE Neurotox Assay, 40 CFR 798.6540 Acute Delayed Neurotoxicity of Organophosphorus Compounds, 40 CFR 798.6560 Subchronic Delayed Neurotoxicity of Organophosphorus Compounds; OPP 81–7, OPP 82–6 Delayed Neurotoxicity of Organophosphorus Substances Following Acute and 28-Day Exposures (Pesticide Assessment Guidelines, Subdivision F—Hazard Evaluation: Human and Domestic Animals, Addendum 10, EPA report 540/09–91–123, March 1991); OECD 418 Delayed Neurotoxicity of Organophosphorus Substances following Acute Exposure and OECD 419 Delayed Neurotoxicity of Organophosphorus Substances: 28-Day Repeated Dose Study.

(b) **Purpose.** In the assessment of organophosphorus substances, (OPs) studies of delayed neurotoxicity using the adult hen as the test animal and including behavioral observation of gait, histopathological assessment of brain, peripheral nerve, and spinal cord, and neurochemical assessment of inhibition of acetylcholinesterase (AChE) and neurotoxic esterase (NTE) are needed to identify and characterize these potential effects.

(c) **Definitions.** The definitions in section 3 of the Toxic Substances Control Act (TSCA) and the definitions in 40 CFR Part 792—Good Laboratory Practice Standards apply to this test guideline. The following definitions also apply to this test guideline.

ED is the effective dose.

LD50 is the median lethal dose.

Neuropathy target esterase (NTE) or neurotoxic esterase is a membrane-bound protein that hydrolyzes phenyl valerate. The inhibition and ‘‘aging’’ of the phosphorylated NTE, i.e. the covalent binding of the OP to the enzyme, is highly correlated with the initiation of organophosphorus induced delayed neurotoxicity (OPIDN). Not all OPs that inhibit NTE cause OPIDN, but all OPs that cause OPIDN inhibit NTE.

NOEL is the no-observed-effect-level.

NTE activity is operationally defined as the phenyl valerate hydrolytic activity resistant to paraoxon (diethyl 4-nitrophenyl phosphate) but sensitive to mipafox (*N*,*N*′-diisopropylphosphorodiamido fluoridate) or neuropathic OP ester inhibition.

Organophosphorus induced delayed neurotoxicity (OPIDN) is a neurological syndrome in which limb weakness and upper motor neuron spasticity are the predominant clinical signs; distal axonopathy of peripheral nerve and spinal cord are the correlative pathological signs, and inhibition and aging of neurotoxic esterase in neural tissues are the correlative biochemical effects. Clinical signs and pathology first appear between 1 and 2 weeks following exposures that typically inhibit and subsequently age neurotoxic esterase.

(d) **Principle of the test method.** The test sequence consists of acute and 28–day exposure studies. Any significant effects on behavior (delayed effects), histopathology, or inhibition of NTE in the acute study are sufficient cause to conduct the 28–day study. The test substance is administered orally to domestic hens that in some cases have been protected from acute cholinergic effects. The animals are observed for at least 21 days after the last dose for gait changes and other signs. Neurochemical examination of selected neural tissues is undertaken on some animals at some times after exposure. Histopathology of brain, spinal cord, and peripheral nerve are performed at the termination of 21–day observation periods. If the results of the acute study are completely negative, that is, there are no delayed behavioral or histopathological effects, and no significant NTE inhibition, the 28–day study is not required. Otherwise, the 28–day study should be conducted. In the 28–day study, three exposure levels are used to describe the dose response curve sufficiently to estimate a reference dose.

(e) **Test procedures**—(1) **Animal selection.** The adult domestic laying hen (*Gallus gallus domesticus*), aged 8 to 14 months, is recommended. Standard size breeds and strains should be employed. Healthy young adult hens free from interfering viral diseases and medication and without abnormalities of gait should be acclimatized to the laboratory conditions for at least 5 days prior to randomization and assignment to treatment and control groups.

(2) **Housing and feeding conditions.** Cages or enclosures which are large enough to permit free mobility of the hens and easy observation of gait should be used. Where the lighting is artificial, the sequence should be 12 h light/12 h dark. Appropriate diets should be administered as well as an unlimited supply of drinking water. The hens should be weighed weekly. Any moribund hens should be removed and sacrificed.

(3) **Route of administration.** Dosage of test substance should normally be by the oral route, preferably by gavage. Liquids may be given neat or dissolved in an appropriate vehicle such as corn oil; solids should be dissolved if at all possible since large doses of solids in gelatin capsules may significantly impair absorption. Dermal exposures may be the most significant route of exposure for applicators and for nonfood uses and there

may be important differences in toxicity by this route. Conduct of these studies by this route may be appropriate and should be considered.

(4) **Study design**—(i) **General.** An important consideration for the design of these studies is prediction of activity based on the structure of the material and the published literature. Some materials, e.g. phosphinates, are known to inhibit NTE, but not to be capable of aging and thus are not expected to cause OPIDN. Many materials have structural features that will permit inhibition and aging, i.e. ester linkages, and are of potential concern. Published data are available for many materials and may be very useful for many aspects of the design and interpretation of these studies.

(ii) **Dose levels and selection.** For the acute study, a single exposure group is required. The acute dose level should be chosen to maximize the amount of material given to the hens, particularly in cases where some activity is expected. For the 28–day study, at least three exposure groups are required in addition to the vehicle control group. Ideally, the data should be sufficient to produce a dose-effect curve. The use of equally spaced doses and a rationale for dose selection that will maximally support detection of dose-effect relations is strongly encouraged. The rationale for dose selection chosen by the investigator should be explicitly stated. The following guidance for dose selection is somewhat complex and is not intended to be followed rigidly.

(A) **Acute study.** Selection of the dose level for the acute study may be based on a limit dose or lethal doses and other available data, e.g. on NTE inhibition.

(*1*) Levels of test substances greater than 2 g/kg need not be tested.

(*2*) Lethal doses. Either an LD50 or an approximate lethal dose (ALD) in the hen may be used to determine the acute high dose. If, from the preliminary data, cholinergic signs are seen very soon after dosing, prophylaxis using atropine may be appropriate. Atropine (20 mg/kg, s.c., up to every 2 h) should be used to prevent death from acute cholinergic effects.

(B) **28–day study.** (*1*) Levels of test substances greater than 1 g/kg need not be tested.

(*2*) High dose—the high dose selected should be estimated to be sufficient to cause OPIDN or be a maximum tolerated dose based on the acute data, but not result in an incidence of fatalities that would prevent a meaningful evaluation of the data.

(*3*) Low dose—the low dose should be estimated to be a minimum effect level, e.g. an ED10, or alternatively, a NOEL.

(*4*) The intermediate dose level should be equally spaced between the high and low doses.

(*5*) Intermediate responses in NTE i.e. greater than 15 percent and less than 70 percent, can be crudely extrapolated as if the dose-response were a simple first order relationship. That is, if a certain dose caused 50 percent inhibition, twice that dose might cause 75 percent inhibition. Such extrapolation is very crude but can be useful in giving some guidance for dose estimation.

(iii) **Numbers of animals.** Exposure groups should be large enough to provide six survivors for both behavioral observations and histopathology. At least three hens are required for determination of NTE in each dose or control group and at each time point.

(iv) **Control groups.** A positive control group of at least six hens treated with a known delayed neurotoxicant, such as tri-*ortho*-cresyl phosphate (TOCP), is required for both acute and 28–day studies. This group may be a concurrent or historical control group. (This should also include at least three hens assessed for biochemical measurements.) Periodic redeterminations of the sensitivity of the assays is suggested, for historical control data, i.e. when some essential element of the test conduct by the performing laboratory has changed. A concurrent control group sufficient to provide six survivors for histopathology and three hens for NTE measurement are treated in a manner identical to the treated groups, except that administration of the test substance is omitted. When protective agents are used, all members of the dose groups and vehicle controls should receive the same treatment.

(5) **Study conduct**—(i) **Biochemical measurements**—(A) **NTE assay.** The test method is a differential assay of the ability of neural tissue, following OP exposure, to hydrolyze a phenyl valerate substrate selectively. The principle of the assay is first to determine the amount of hydrolysis that occurs in the presence of a nonneurotoxic inhibitor, paraoxon, (*a*), which is intended to occupy irrelevant sites, and second to determine the activity in the presence of paraoxon and a known neuropathic inhibitor, mipafox, (*b*). NTE activity is the difference between (*a*) and (*b*), that is, the proportion of activity inhibited only by mipafox. Thus, the ‘‘mipafox site’’ is already occupied following exposure to a neuropathic OP ester and the activity of (*b*) is therefore reduced.

(*1*) Three hens from each group should be sacrificed at 48 h after the last dose. Depending on the duration of acute signs as an indication of the disposition of the test material, the time for sacrifice for NTE and AchE assessment may be chosen at a different time to optimize detection of effects. Both the brain and spinal cord should be prepared for assay of NTE. Perform duplicate assays of NTE in brain and spinal cord of three birds from each group and control group.

(*2*) Materials. This assay requires paraoxon, mipafox, and phenyl valerate, all of which can be obtained commercially.

(*3*) The assay has four stages: Preparation of tissue, differential preincubation, hydrolysis of substrate, and measurement of product. The quotations that follow are from Johnson under paragraph (g)(7) of this guideline as corrected or modified in paragraph (g)(9) of this guideline. His is the best known method for conduct of this assay. Other acceptable methods, which primarily involve minor technical modification, have been used (see paragraphs (g)(13) and (g)(14) of this guideline).

* * * the whole brain (is) removed and cooled in ice-cold buffer (50 mM Tris/0.2 mM EDTA adjusted to pH 8.0 at 25 °C with HCl). Meninges and blood vessels are rapidly removed and the brain is blotted dry, weighed, and homogenized thoroughly in ice-cold buffer (at a volume of at least 1:30, W/V), using a high-speed rotating perspex pestle with not more than 0.25 mm difference in diameter between pestle and tube.

Paired samples of homogenate (equivalent to about 6.0 mg tissue) are preincubated in Tris/EDTA buffer pH 8 at 37 °C for exactly 20 min with paraoxon (40 to 100 μM) plus either (a) buffer or (b) mipafox (50 μM) in a final volume of 2 mL.

After preincubation, dispersion (2 mL) of phenyl valerate is added and the incubation is continued for exactly 15 min. The dispersion is prepared by adding a solution of Triton X–100 (0.03 percent in water) (30 vol) to a solution of phenyl valerate (15 or 20 mg/mL) in redistilled dimethylformamide (1 vol) and mixing thoroughly (by swirling): other solvents give less satisfactory dispersions. Reaction is stopped by adding 2 mL of sodium dodecyl sulfate (1–2 percent W/V) in buffer containing 4-aminoantipyrine (otherwise known as 4-aminophenazone) (0.25 percent).

This assay is based on the colorimetric determination of liberated phenol. The coupling of phenol liberated in the assay with the aminoantipyrine may be performed at any convenient time after quenching the enzyme: 1 mL of $K_3Fe(CN)_6$ (0.4 percent in water) is added and the stable red colour is read at 490 nm.

A nontissue blank, kept to 10 percent of the paraoxon tube value by maintaining the substrate phenol-free, should be included in each group of assay tubes. Typical control absorbance values would be 0.8 for paraoxon, 0.35 for paraoxon and mipafox and 0.07 for the blank. Colour development takes 1–2 min in solutions stopped with sodium dodecyl sulphate. The extinction coefficient of phenol under these conditions is 15,600 at a wavelength of 490 nm. NTE activity is represented by the difference in absorbance obtained from samples incubated under conditions (*a*) and (*b*) respectively.

Under standard conditions NTE hydrolyzes about 2,400 nmol substrate/min/g of cortex, 550 for spinal cord, and 100 for sciatic nerve. * * *

(B) **AChE measures.** Assay of acetylcholinesterase in the brains of the same birds (according to paragraphs (g)(3) and (g)(4) of this guideline) should also be performed. The level of AChE inhibition is a useful index of lethal potency and the ratio of lethal potency to NTE inhibitory potency can be useful for subsequent dose selection.

(ii) **21–Day observation.** All remaining hens should be carefully observed at least once daily for a period of at least 21 days until 21 days after the last dose and signs of toxicity recorded, including the time of onset, degree, and duration. Observations should include, but not be limited to, behavioral abnormality, locomotor ataxia, and paralysis. At least twice a week the hens should be taken outside the cages and subjected to a period of forced motor activity, such as ladder climbing, in order to enhance the observation of minimal responses. A rating scale of at least four levels should be used to grade ataxia (see paragraph (g)(12) of this guideline).

(iii) **Necropsy and histopathology.** (A) Gross necropsies are recommended for all survivors and should include observation of the appearance of the brain and spinal cord. All animals should be prepared for microscopic examination. Tissues should be fixed by whole body perfusion, with a fixative appropriate for the embedding media. Sections should include medulla oblongata, spinal cord, and peripheral nerves. The spinal cord sections should be taken from the rostral cervical, the midthoracic, and the lumbosacral regions. Section of the proximal regions of both of the tibial nerves and their branches should be taken. Sections should be stained with appropriate myelin- and axon-specific stains.

(B) For 28–day studies, a stepwise examination of tissue samples is recommended. In such a stepwise examination, sections from the high dose group are first compared with those of the control group. If no neuropathological alterations are observed in samples from the high dose group, subsequent analysis is not required. If neuropathological alterations are observed in samples from the high dose group, samples from the intermediate and low dose groups are examined sequentially.

(f) **Data reporting and evaluation**—(1) **Test report.** In addition to any other applicable reporting requirements, the final test report must include the following information:

(i) Toxic response data by group with a description of clinical signs; the criteria for the grading system for ataxia and any other scales should be defined.

(ii) For each animal, time of death during the study or whether it survived to termination.

(iii) The day of the first occurrence of each abnormal sign and its subsequent course including its degree.

(iv) Body weight data.

(v) Necropsy findings for each animal, including a description of the appearance of the brain and the spinal cord.

(vi) Biochemical data for each animal assessed, including absorbance values for each animal tested, and blank sample data.

(vii) A detailed description of all histopathological findings.

(viii) Statistical treatment of results, where appropriate.

(2) **Treatment of results.** Data may be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions or effects, the types of lesions or effects and the percentage of animals displaying each type of lesion or effect.

(3) **Evaluation of results.** The findings of these delayed neurotoxicity studies should be evaluated in terms of the incidence and severity of behavioral, neurochemical, and histopathological effects and of any other observed effects in the treated and control groups, as well as any information known or available to the authors, such as published studies. For a variety of results seen, further studies may be necessary to characterize these effects.

(g) **References.** The following references should be consulted for additional background information on this test guideline:

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(13) Soliman, S.A. et al. Species Susceptibility to Delayed Toxic Neuropathy in relation to in vivo inhibition of Neurotoxic Esterase by Neurotoxic Organophosphorus Ester. *Journal of Toxicology and Environmental Health* 9:189–197 (1982).

(14) Sprague, G.L. et al. Time course for neurotoxic esterase activity in hens given multiple diisopropyl fluorophosphate injections. *Neurotoxicology* 2:523–532 (1981).